REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS AND AMENDMENTS

Claims 2-7 were pending in this application when last examined and stand rejected.

Claims 2 and 6 are amended to clarify that the soluble outer membrane protein is the soluble F3 protein. Support can be found in the disclosure, for example, at page 7, lines 25-27, and in Example 1 on pages 13-14. The soluble outer membrane protein of the present invention is F3 from the merozoite of *Eimeria acervulina*, which is a fraction of soluble outer membrane proteins of 18 to 27kD and has a common immunogenicity shared among sporozoite and merozoite of *Eimeria acervulina*, *Eimeria tenella* and *Eimeria maxima* which are associated with chicken coccidiosis. Claims 2 and 6 are also amended to change "a" to "the" before "soluble outermembrane protein F3." Support can be found in the claims as filed.

No new matter has been added.

II. INFORMATION DISCLOSURE STATEMENT

In item 2 on page 2 of the non-final Office Action of March 30, 2007, it was indicated that reference JP 60-72827 (designated AL) in the IDS of March 2, 2007 was not considered on the basis the submitted Abstract is for EP 0135073 and not for said Japanese patent.

As noted in item II on page 4 of the Amendment filed June 4, 2007, Applicants again respectfully submitted that JP 60-72827 should have been considered, because the English Abstract for EP 0135073 is based on the European counterpart application of JP 60-72827. Pursuant to M.P.E.P. § 609.04(a)III, it is sufficient for Applicants to submit an English Abstract for corresponding related applications as a brief summary of the Japanese application. See attached M.P.E.P. pages 600-153 to 600-154.

Therefore, Applicants again respectfully request the Office to consider JP 60-72827 and return an Examiner-initialed PTO-1449 form indicating such.

III. WRITTEN DESCRIPTION & ENABLEMENT REJECTIONS

Claims 2-7 were again rejected under 35 U.S.C. § 112, first paragraph, as lacking written description support for the claimed invention for the reasons set forth in item 3 on pages 2-10 of the final Office Action.

Claims 2-7 were again rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement for the claimed invention for the reasons set forth in item 4 on pages 10-16 of the Action. The basis for this rejection is essentially the same as that for the above-discussed 112, first paragraph, enablement rejection. Accordingly, these rejections will be discussed together below.

Applicants respectfully traverse these rejections.

First, the Office indicated that the Rule 132 Declaration by Dr. Kodama and the arguments in the response filed June 8, 2007 were unpersuasive, because the word "a" in the term "a soluble membrane protein" coupled with the molecular weight range of "18 to 27 kD" in the claims implies that there is more than one protein to choose from and the F3 protein is not the only protein being described. Accordingly, the Office contends that the claims read on "any" soluble membrane protein. See the top of pages 4 and 6.

In reply, claims 2 and 6 have amended to clarify that the soluble outer membrane protein is <u>the</u> <u>soluble protein F3</u>. Claims 2 and 6 are also amended to change "a" to "the" before "soluble outermembrane protein F3." Consequently, the soluble outer membrane protein of the present invention is the soluble protein F3 from the merozoite of *Eimeria acervulina*, which is a fraction of soluble outer membrane proteins of 18 to 27kD and has a common immunogenicity shared among sporozoite and merozoite of *Eimeria acervulina*, *Eimeria tenella* and *Eimeria maxima* which are associated with chicken coccidiosis. Support for the soluble outer membrane protein is the soluble F3 protein can be found in the disclosure, for example, at page 7, lines 25-27. See also Example 1 on pages 13-14. Thus, Applicants respectfully submit that the amended claims are not directed to a vast genus of antigenic outer membrane proteins. Again, the F3 protein is the only protein being claimed and the F3 protein is a fraction of soluble outer membrane proteins of 18 to 27 kD.

Further, as noted in the last response, the soluble membrane protein F3 protein was well known in the art field prior to Applicants' priority date. See page 7, lines 25-27, and Example 1 on pages 13-14 of the disclosure. At these locations, the specification makes reference to Lillehoj et al. (Avian Diseases, Vol. 44, pp. 379-389, April 2000). This reference describes how to prepare the F3 protein. This reference was submitted to the Office in the IDS of March 6, 2006 and a courtesy copy was attached to the last response.

Please see the description in the section, "Preparation of rabbit antisera" in the paragraph bridging pages 380 and 381 of this reference, where it clearly describes that an 18- to 29- kD fraction of *Eimeria acervulina* merozoite soluble antigen (F3) was obtained by preparative SDS-PAGE. The reference further describes rabbit antiserum against an 18- to 27-kD native protein fraction (F3) from *Eimeria acervulina* merozoites. See the Abstract on page 379.

As such, Applicants again respectfully submit that: (1) the protein used to immunize to obtain the antibody in the present invention was known and fully characterized in the art; and (2) the skilled artisan could obtain the protein F3 based on the instant disclosure and the knowledge in the art.

In addition, the specification provides a working example (Example 1 on pages 13-14) describing an experiment to obtain the soluble outer membrane protein of 18 to 27 kD. This Example describes production of the claimed antibody. The F3 is a fraction of 18- to 27-kD proteins. Thus, Applicants have demonstrated possession by an actual reduction to practice of the present invention for both the claimed antibody and the antigen used to immunize to obtain the antibody.

Thus, Applicants again respectfully submit that: (1) the claims do not encompass a vast number of antigenic outer membrane proteins; and (2) the F3 antigen used to immunize and obtain the antibody of the present invention was well known and fully characterized in the art.

Based on the above, it is respectfully submitted that the specification provides adequate written description support to show that Applicants were in possession of the claimed invention at the time of filing. For these reasons, the 112, first paragraph, written description and enablement rejections of claims 2-7 are untenable and should be withdrawn.

Second, on page 6 of the Action, the Office argued that the rejection was maintained, because the claims are not commensurate in scope with the data in the Rule 132 Declaration by Dr. Kodama. Specifically, the Office indicated that the declaration does not provide support for "any" antibody raised against any soluble outer membrane.

In reply, Applicants note that the amended claims overcome this concern as the claims are limited to the soluble outer membrane protein F3 for the reasons discussed above. As such, the claims are not directed to just any antibody, but to those generated against the soluble outer membrane protein F3

Third, the Office argued that the skilled artisan cannot conclude from the data in the declaration that the chick is protected against coccidiosis due to a change in average weight, rate of maturity and productivity. See the top of page 7 of the final Office Action.

Applicants respectfully traverse this position.

Applicants previously submitted two Rule 132 Declarations on June 8, 2007 and December 22, 2006 as evidence of the therapeutic effectiveness of the claimed composition in inducing protective immunity against chicken coccidiosis. Applicants cannot understand why the Office is unsatisfied with the data presented in the Declarations.

The Rule 132 Declaration by Dr. Kodama attached to the June 8, 2007 response sufficiently demonstrates that the claimed composition is capable of inducing protective immunity against chicken coccidiosis. Item 2 on page 1 of the Declaration describes that the anti-chicken coccidiosis antibody produced in Example 1 of the present invention was administered in feed to chickens. After administration, the chickens were infected with oocysts of *Eimeria tenella*. As a control, the chickens which had been administered the standard broiler feed without the antibody and were not infected were also observed.

As described therein, "When Chicks are affected with coccidiosis, average weight and rate of maturity will greatly decrease, as a result, productivity will greatly decrease." The chicks administered with the antibody of the present invention showed a high rate of maturity (94.6% or more), although they had been raised in the contaminated farm. If chicks are raised in a

contaminated farm without any agent, such a high rate of maturity would not be achieved, and most of the chicks would die. This explanation is simple and clear.

Thus, Applicants respectfully submit that the Declaration attached to the June 8, 2007 response clearly demonstrates that administration of the claimed antibody before infection resulted in ***significantly improved*** conditions of the chickens after infection, including improvements in weight gain and feed conversion ratio. Accordingly, the Declaration clearly proves the claimed antibody to be very effective in preventing chicken coccidiosis. See Table 1 on page 2 of the Declaration.

Nonetheless, Applicants have attached herewith a relevant journal article (Zootecnica, pp. 48-52, November 2001) that supports the position set forth above with regards to the data in the Declaration. This reference supports Applicants' position that growth and improvements in weight gain and feed conversion ratio are relevant indicators for successful therapy. See for instance, the disclosure in the 1st column on page 48.

Thus, Applicants again respectfully submit that Declaration submitted with the June 8, 2007 response and the Declaration submitted with the response filed December 22, 2006 provide sufficient experimental evidence demonstrating the therapeutic effectiveness of the claimed composition in inducing protective immunity against chicken coccidiosis.

For these reasons, the above 112, first paragraph, written description and enablement rejections of claims 2-7 are untenable and should be withdrawn.

IV. OBVOIUSNESS REJECTION

Claims 2, 4, and 6 were newly rejected under 35 U.S.C. § 103(a) as obvious over Lillehoj et al. (Avian Diseases, vol. 44, pp. 379-389 (2000) (of record) in view of Wells et al. (Antonie van Leeuwenhock, vol. 70, pp. 317-339 (1996) (copy is enclosed) for the reasons in item 5 on pages 16-18 of the Office Action.

Applicants respectfully traverse this rejection.

The Office indicated that Lillehoj et al. discloses oral immunization of chickens with F3 protein. Applicants respectfully submit that the Office's characterization of the reference is incorrect.

Lillehoj et al. discloses an 18- to 27-kD native protein fraction (F3) from Eimeria acervulina merozoites and a monoclonal antibody against the protein. However, Lillehoj et al. only discloses oral immunization of chickens with the F3 protein (i.e., the antigen) itself. Lillehoj et al. never describe oral administration of the antibody against the antigen. It is described on page 385, left column, 13th line from the bottom that "in experiment, 3-wk-old chickens were twice immunized orally…with…recombinant 3-1 protein, then challenged…with…E. acervulina oocysts." In this experiment, the chickens were orally administered not with antibody but with 3-1 protein (F3 protein).

The Office seems to confuse an oral administration (immunization) of F3 protein disclosed by Lillehoj et al. and an oral administration of the antibody against F3 protein in the present invention. That is, Lillehoj et al. discloses an active immunity (cellular immunity), but the present invention relates to an oral passive immunity (humoral immunity). These are vastly different forms of immunity. Please see for example the following web sites: (http://en.wikipedia.org/wiki/Passive_immunity) and (http://en.wikipedia.org/wiki/Cellular_immunity), which highlighted the clear distinctions of such as known in the art.

It was a common general technical knowledge in this field that the passive immunity was quite different from the active immunity both in procedure and mechanism. It was believed that only active immunity was effective for coccidiosis and passive immunity was not effective before the present invention as evidence by the attached Lillehoj reference (Infection and Immunity, Vol. 55, No. 7, pp. 1616-1621, July 1987). It is respectfully submitted that one of ordinary skill in the art, upon reading this reference and the cited prior art references, would reasonably conclude that the state of the art was such that passive immunity would not be an effective treatment for avian coccidiosis. See, for instance, page 1616, 1st column, 1st paragraph, starting at line 10.

Based on this knowledge in the art and the lack of a suggestion in the cited references, there is no reasonable expectation of success to modify the cited prior art teachings to arrive at the claimed invention.

Applicants found for the first time that chicken coccidiosis can be effectively prevented and treated by orally administering the chicken egg antibody (polyclonal antibody) of the present invention (passive immunity).

For this reason, Applicants respectfully submit that the present invention is novel and unobvious over the combined cited references. Therefore, the above-noted 103(a) obviousness rejection is untenable and should be withdrawn.

V. CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

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December 11, 2007

ATTACHMENTS

1. Lillehoj, Infection and Immunity, Vol. 55, No. 7, pp. 1616-1621, July 1987; and
2. Zootecnica, pp. 48-52, November 2001.

INFECTION AND IMMUNITY, July 1987, p. 1616–1621 Vol. 55, No. 7
0019-9567/87/071616-06$02.00/0

Effects of Immunosuppression on Avian Coccidiosis: Cyclosporin A but Not Hormonal Bursectomy Abrogates Host Protective Immunity

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The effects of cyclosporin A (CsA) treatment and hormonal bursectomy on *Eimeria tenella* infection of chickens were investigated to evaluate the role of humoral antibody and cell-mediated immunity (CMI) in the host protective immunity to an intestinal protozoan disease, coccidiosis. Hormonal bursectomy had no significant effect on the host response to *E. tenella*. CsA treatment had a differential effect on the course of disease depending on how CsA was given relative to infection. Daily administration of CsA for 7 days beginning 1 day before primary infection with *E. tenella* enhanced disease resistance, whereas a single dose of CsA given before primary infection enhanced disease susceptibility compared with that of untreated controls. Chickens treated with CsA during the primary infection were resistant to reinfection at 5 weeks post-primary infection. Treatment of chickens immune to *E. tenella* with CsA at the time of secondary infection abrogated their resistance to reinfection despite the presence of high levels of coccidia-specific secretory immunoglobulin A and serum immunoglobulin G. Splenic lymphocytes obtained after CsA treatment demonstrated a substantially depressed concanavalin A response, but not a depressed lipopolysaccharide response. Because CsA was not directly toxic to parasites in vivo when administered during the secondary infection, these results suggest that CsA interacts with the immune system to allow priming during the primary infection, while interfering with the effector function of CMI during the secondary infection. Taken together, present findings indicate that CMI plays a major role in host protective immunity to *E. tenella*.

Coccidiosis is an intestinal protozoan infection caused by any of eight species of the genus *Eimeria*. Much work has characterized the immune response elicited by *Eimeria* spp. and identified the means by which such responses are translated into protective immunity. Although earlier investigations implicated the role of humoral factors in antiparasitic immunity (reviewed in reference 22), recent investigations in agammaglobulinemic chickens (7; H. S. Lillehoj, Poult. Sci. 65:80, 1986) suggest that antibodies do not play a major role in the development of protective immunity. The importance of cell-mediated immunity (CMI) in the development of protective immunity to coccidiosis has been suggested in mice (20), but there is, as yet, no convincing in vivo model system to study the role of T-cell-mediated immunity in avian coccidiosis.

Cyclosporin A (CsA), a neutral, hydrophobic, cyclic, fungal metabolite, is a well-known immunosuppressive drug that interferes at an early stage with the activation of resting lymphocytes by inhibiting the expression of the gene for interleukin-2 (14, 16), the expression of the interleukin-2 receptor antigen (18), and the production of other lymphokines (24). The early studies of Borel and co-workers (2) clearly established that CsA is an immunosuppressive agent that selectively acts on a defined subpopulation of immunocompetent T cells. Much of the information concerning the action of CsA is based on in vitro experimental systems; thus, the immunoregulatory role of CsA in vivo has not been thoroughly investigated. Recent suggestions that CsA has a different mode of action in vivo than in vitro (13) compel further investigation on the mechanism of immunomodulation mediated by CsA in vivo.

The present study was undertaken to investigate the immunoregulatory role of CsA in vivo and the effect of CsA treatment and hormonal bursectomy on primary and secondary infections with *Eimeria tenella*. Two different inbred strains of chickens, genetically showing different disease susceptibility to *E. tenella* (19), were used to rule out the possibility that the CsA-mediated immunomodulation of coccidial infection was dependent on the host genetic background. Three different regimens of CsA treatment were investigated: (i) administration during only the preinfection phase, (ii) administration during only the induction and clinical phase of immunity, and (iii) administration only on the established acquired resistance. This report describes the differential immunological effects that CsA has on the primary and secondary infections with *E. tenella* depending on the treatment regimen.

MATERIALS AND METHODS

Chickens. Embryonated SC (B2/B2) and FP (B15/B21) chicken eggs were obtained from Hy-line International Production Center (Dallas Center, Iowa). All chickens were housed in clean wire-floored cages and provided feed and water ad libitum. Chickens that were 4- to 6-weeks-old were used in all experiments.

Development of agammaglobulinemic and dysgammaglobulinemic chickens. Hormonal bursectomy was done by combining in ovo treatment with testosterone propionate (Sigma Chemical Co., St. Louis, Mo.) and cyclophosphamide (Sigma) injection at hatching as described previously (8). On day 12 of embryonation, each egg was injected in the allantoic sac with 0.1 ml of sterile corn oil containing 4.0 mg of testosterone propionate. At 1 and 2 days of age, each chick was intraperitoneally injected with 4.0 mg of cyclophosphamide dissolved in ethanol-saline. Hormonally bursectomized chickens were tested for the presence of serum immunoglobulin G (IgG), IgM, and IgA at 4 weeks of age by Ouchterlony immunodiffusion and enzyme-linked immunosorbent assay (ELISA) as described below. Agammaglobulinemic chickens with no detectable immunoglobu-

lin were unable to produce anticoccidial antibodies upon infection with *E. tenella*. Dysgammaglobulinemic chickens were deficient in at least one subclass of immunoglobulin and produced anticoccidial antibody of only certain subclasses upon infection with *E. tenella*.

Parasites and inoculation of birds. The strain of *E. tenella* used (LS24) was developed from a single oocyst isolation and maintained at the Animal Parasitology Institute (Beltsville, Md.). Ten 4-week-old chickens were inoculated orally with 10^4 sporulated oocysts of *E. tenella*. This inoculating dose gave an optimum oocyst production in both SC and FP chickens in the previous study (H. S. Lillehoj, Poult. Sci. 65:79, 1986). Four consecutive daily oocyst counts for individual birds began 5 days post-primary inoculation (p.p.i.) using a McMaster counting chamber. Because a previous investigation showed that chickens develop protective immunity starting at 3 weeks p.p.i. (17), secondary inoculation was done by inoculating immune birds with 10^4 oocysts at 5 weeks p.p.i. Four consecutive daily oocyst counts were made in the individual birds beginning 6 days post-secondary inoculation (p.s.i.).

Lesion score and PCV determination. Chickens show severe cecal lesions and a significant decrease in packed-cell volume (PCV) upon infection with *E. tenella*. Cecal lesion score was assessed by the previously described method (10) at 5 days p.p.i. PCV was determined at 5 days p.p.i. in birds infected with *E. tenella* as described previously (19).

ELISA. The previously described procedure for ELISA (19) and the optimum concentrations of antigen, antisera, and substrate were used to assess antibody response to coccidia.

To determine the presence of immunoglobulins in chickens that were hormonally bursectomized, I tested sera obtained from chickens in the ELISA. Antigen-coated plates were prepared by coating plates with optimum concentrations of rabbit anti-chicken IgG or goat anti-chicken IgM or IgA specific for each of the heavy chains of chicken immunoglobulins (Miles Laboratories, Inc., Elkhart, Ind.).

Antigen-coated plates were treated with 10% bovine serum albumin for 2 h at room temperature to block nonspecific binding sites. Serum to be tested was serially diluted twofold. A 50-μl sample of chicken serum to be tested was added to antigen-coated wells. After 2 h of incubation at room temperature, the plates were washed four times with the washing buffer (phosphate-buffered saline containing 0.05% Tween 20). Rabbit anti-chicken IgG (50 μl) (Miles) was added, and plates were incubated for 1 h at 37°C. The plates were washed four times and then incubated with 50 μl of biotin antirabbit antiserum (Sigma). After 30 min of incubation at 37°C, the plates were washed and incubated with 50 μl of streptavidin-peroxidase (Zymed, San Francisco, Calif.). Enzyme reactions were initiated by the addition of *ortho*-phenylenediamine dihydrochloride dissolved in 0.05 M citrate phosphate buffer containing fresh H_2O_2. Optical density was read at 450 nm with a multichannel spectrophotometer (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.).

In vivo treatment with CsA. To investigate the effects of immunosuppression of CMI on the host response to coccidia, I treated chickens with CsA. CsA dissolved in olive oil at 100 mg/ml was used to treat chickens. Two different regimens were used to investigate the effect of CsA treatment on the primary infection of *E. tenella*. In one group, chickens were treated 1 day before primary infection with 0.5 ml of CsA per os, followed by infection with either 10^5 or 10^4 sporulated oocysts of *E. tenella*. Another group of chickens was treated with 0.5 ml of CsA for 7 consecutive days beginning 1 day before primary infection. Controls were inoculated with olive oil alone. To investigate the effect of CsA on secondary infection, I treated chickens daily with 0.5 ml of CsA for 2 weeks beginning 1 day before secondary inoculation. These chickens were challenged with 10^4 sporulated oocysts of *E. tenella* at 5 weeks p.p.i. Each group consisted of six 4-week-old SC and FP chickens.

In vitro mitogen-induced lymphoproliferation assay. Single-cell suspensions were prepared from spleens obtained from control or CsA-treated chickens by teasing spleen cells in Hanks balanced salt solution. Mitogen-induced stimulation was performed by incubating various concentrations of splenic lymphocytes with different concentrations of concanavalin A (ConA) (Pharmacia, Inc., Piscataway, N.J.) or lipopolysaccharide (LPS) (Sigma) in 96-well microtiter plates in 0.2 ml of complete medium for 3 days. Complete medium consisted of RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5} M), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (10 mM), and 5% fetal calf serum. Cell cultures were incubated at 41°C in a humidified atmosphere of 6% CO_2 in air. Twenty hours before harvesting, 1 μCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.) was added to each well. The cultures were harvested with a PHD cell harvester (Cambridge Technology, Inc., Cambridge, Mass.), and the amounts of radioactivity associated with cellular DNA were measured with a β-scintillation counter (Packard Instruments Co., Inc., Rockville, Md.).

Statistics. The Mann-Whitney U test (25) was used to test for differences among groups.

RESULTS

Effect of hormonal bursectomy on primary infection with *E. tenella*. To determine the effect of immunosuppression of humoral response on avian coccidiosis, I rendered chickens B-cell deficient by hormonal bursectomy. The efficiency of hormonal bursectomy depended on the dose and treatment regimens. In general, treatment of SC and FP chickens with testosterone in ovo and with cyclophosphamide at hatching resulted in agammaglobulinemia in only 10% of hormonally bursectomized chickens (data not shown). These agammaglobulinemic chickens did not produce anticoccidial antibodies at 14 days p.p.i., suggesting that they were truly agammaglobulinemic (data not shown). A total of 50% of hormonally bursectomized chickens lacked one or two immunoglobulin isotypes. The remaining 40% had detectable IgG, IgM, and IgA and produced high levels of anticoccidial antibodies upon infection with *E. tenella*. Normal and immunoglobulin-deficient chickens (4 weeks old) were infected with 10^5 oocysts of *E. tenella*, and disease susceptibility was assessed by determining the PCV at 5 days p.p.i. (Table 1). PCV was measured since a previous investigation showed that it provides a sensitive measurement of the severity of infection (19). In general, SC chickens that were hormonally bursectomized (group 2) had lower PCVs than untreated chickens (group 1) despite the presence of all three types of immunoglobulins (Table 1) ($P < 0.05$, Mann-Whitney U test; level of significance for two-tailed test, $\alpha = 0.05$). Agammaglobulinemic (group 3) and dysgammaglobulinemic (groups 4, 5, and 6) chickens did not differ significantly with respect to disease susceptibility. These results suggest that B-cell immunodeficiency does not influence the outcome of primary infection with *E. tenella*.

Oocyst production in agammaglobulinemic chickens after primary and secondary infections. Ten 4-week-old normal and agammaglobulinemic chickens were inoculated with 10^4 oocysts, and oocysts were counted to investigate effects of serum and secretory immunoglobulins on disease susceptibility and the acquisition of resistance to reinfection (Fig. 1). A previous study showed that an inoculating dose of 10^4 oocysts gives optimal oocyst production in SC and FP chickens (Lillehoj, Poult. Sci. 65:80, 1986). There were no significant differences in oocyst production between normal and agammaglobulinemic chickens ($P > 0.05$) after primary infection. Furthermore, both normal and agammaglobulinemic chickens were resistant to reinfection upon secondary inoculation on the basis of oocyst production. No significant differences in the duration of the prepatent or patent period were noted between normal and hormonally bursectomized groups, although hormonally bursectomized birds had a higher mortality (data not shown).

Effect of CsA treatment on primary inoculation with *E. tenella*. Because bursectomy studies suggested that humoral immunity does not influence disease, I decided to investigate the effects of CMI on disease using the immunosuppressive drug CsA. To investigate the effects of CsA treatment on the course of coccidiosis upon primary infection, I treated six 4-week-old FP chickens with CsA. Two different treatment regimens were used: one treatment before the inoculation or seven daily treatments starting 1 day before the inoculation. Chickens pretreated once with CsA 1 day before primary infection showed generally produced more oocysts (Fig. 2). In contrast, daily treatment with CsA for 1 week beginning 1 day before primary infection significantly reduced the numbers of oocysts produced ($P < 0.05$). Taken together, these results suggest that prolonged CsA treatment of birds significantly enhances disease resistance, whereas a single pretreatment of birds before primary infection enhances disease susceptibility.

Effect of CsA treatment on secondary infection. To investigate the effects of CsA on the host protective immunity to coccidiosis, I used two different treatment regimens. Groups in Fig. 3 designated as a received daily CsA treatment for 1 week beginning 1 day before the primary infection. Secondary inoculation was given at 5 weeks p.p.i. Groups designated as b received daily CsA treatment for 1 week beginning 1 day before the primary infection and for 2 weeks beginning 1 day before secondary infection. Each group consisted of six 4-week-old chickens. SC and FP chickens not treated with CsA at the time of secondary infection did

TABLE 1. Effect of hormonal bursectomy on primary infection with *E. tenella*

Group	Strain	Treatment[a]	IgG[b]	IgA[b]	IgM[b]	PCV ± SD[c]
1	SC	N	+	+	+	34 ± 1[d]
	FP	N	+	+	+	24 ± 1[e]
2	SC	B	+	+	+	23 ± 2[f]
	FP	B	+	+	+	28 ± 4[e]
3	SC	B	−	−	−	28 ± 5[f]
	FP	B	−	−	−	27 ± 7[e]
4	SC	B	+	−	−	23 ± 6[f]
	FP	B	+	−	−	23 ± 11[e]
5	SC	B	−	+	−	25 ± 1[f]
	FP	B	−	+	−	19 ± 8[e]
6	SC	B	+	+	−	24 ± 1[f]
	FP	B	+	+	−	23 ± 1[e]

[a] Four-week-old normal (N) or hormonally bursectomized (B) chickens (20 per group) were inoculated with 10^3 oocysts of *E. tenella* (LS 24).
[b] The presence of IgG, IgM, or IgA serum immunoglobulin was determined by Ouchterlony immunodiffusion at 4 weeks of age.
[c] PCV was determined at 5 days postinfection. The average results from two independent experiments is shown. Groups with same symbol from the same strain are not statistically different, while those with different symbol are statistically different.

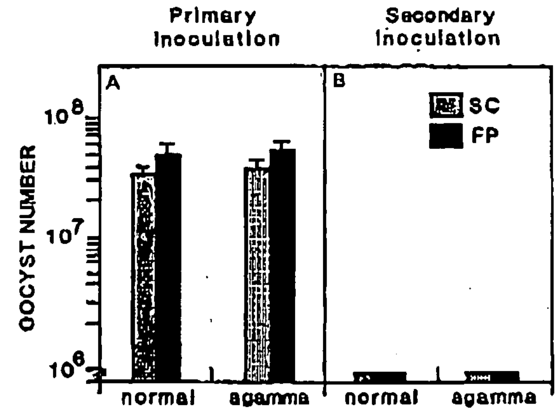

FIG. 1. Oocyst production in normal and agammaglobulinemic chickens upon primary and secondary infection. (A) Ten 4-week-old SC and FP chickens were inoculated with 10^4 sporulated oocysts. Oocyst production was measured during the patent period from days 5 to 10 p.p.i. (B) Ten immune SC and FP chickens were infected with 10^4 sporulated oocysts 5 weeks p.p.i. Oocyst production was measured for 4 days starting 6 days p.s.i.

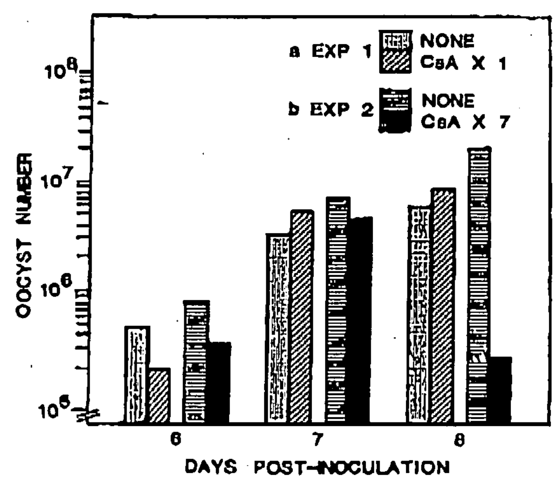

FIG. 2. Effect of CsA treatment on primary infection with *E. tenella*. (a) In experiment 1, six 4-week-old FP chickens were treated with CsA (100 mg/kg of body weight) at 1 day before primary infection. (b) In experiment 2, six 4-week-old FP chickens were treated with CsA for 7 consecutive days starting 1 day before primary infection. Oocyst counts were measured on individual birds beginning 5 days p.p.i. No oocysts were seen during the first 5 days after primary infection.

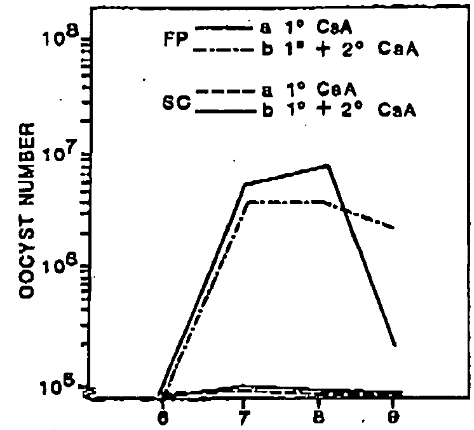

FIG. 3. Effect of CsA treatment on secondary infection with *E. tenella*. Six age-matched SC and FP chickens were infected with 10^4 oocysts of *E. tenella*. Groups designated as a were treated with CsA for 1 week beginning 1 day before primary infection. These birds were reinfected with 10^4 oocysts at 5 weeks p.p.i. No CsA was given to these groups at the time of secondary infection. Groups designated b received CsA treatment at the time of primary and secondary infection. Chickens were treated with CsA for 2 weeks beginning 1 day before secondary infection.

not produce oocysts upon secondary infection. In contrast, chickens treated with CsA during the secondary inoculation produced significant numbers of oocysts at 7, 8, and 9 days p.s.i. No mortality occurred in CsA-treated chickens, indicating that the CsA treatment given in this study was not lethal. Groups of chickens that were not treated with CsA during both the primary and secondary infections were resistant to challenge infection (data not shown). These results suggest that CsA treatment of chickens at the time of secondary infection abrogates the host protective immunity.

Anticoccidial antibodies in CsA-treated birds challenged with *E. tenella*. To assess the effect of CsA treatment on the production of antibodies to *E. tenella*, I tested immune sera and bile secretions obtained from CsA-treated immune chickens using ELISA. Both CsA-treated and non-CsA-treated immune chickens showed high levels of anticoccidial IgG and biliary secretory IgA antibodies (Fig. 4). This result suggests that CsA-mediated abrogation of protective immunity is not due to the immunosuppression of anticoccidial humoral response.

Effect of CsA treatment on ConA- and LPS-induced lymphoproliferation. Since CsA treatment of chickens caused marked modulation of host response to coccidiosis, the effect of peroral treatment of CsA on splenic T- and B-cell function was tested. Chickens treated with CsA once at 1 day before assay or for 1 week beginning at 1 day before assay by daily inoculation of CsA showed a diminished ConA response but not a diminished LPS response (Table 2). Although high concentrations of CsA (up to 1 μg/ml) inhibited the LPS-induced lymphoproliferation response of normal splenic lymphocytes in vitro (data not shown), the lack of CsA inhibition of LPS-induced lymphoproliferative responses of splenic lymphocytes from CsA-treated chickens suggested that the dose of CsA used in the present study was not inhibitory for the LPS-induced B-cell response. These

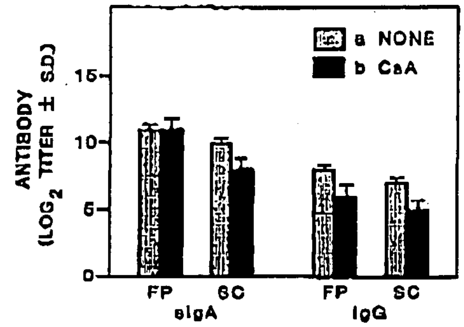

FIG. 4. Anticoccidial antibodies in normal and CsA-treated chickens. FP and SC chickens were infected twice with 10^4 oocysts of *E. tenella* at 5 weeks apart. Chickens in group a received no CsA treatment. Chickens in group b were treated with CsA at the time of primary and secondary infection. Each group consisted of six 4-week-old chickens. Chickens were treated with CsA as described in the legend to Fig. 3. Sera and bile secretions from those chickens were collected at 5 days p.s.i. Secretory IgA (sIgA) and serum IgG were determined by ELISA. S.D., Standard deviation.

results suggest that the dose of CsA used in the present study had a preferential inhibitory effect on T-cell response but not on B-cell response.

DISCUSSION

The results of the present investigation strongly suggest that CMI, rather than humoral immunity, is responsible for the development of a protective immune response against *E. tenella* infection. This conclusion is based on the following observations. (i) Hormonally bursectomized agammaglobulinemic chickens show no difference in oocyst production and PCV upon primary infection or in resistance to secondary infection with *E. tenella*, compared with untreated control chickens. (ii) Oral treatment with CsA caused preferential depression of the splenic mitogenic response to ConA, a T-cell mitogen, but not to LPS, a B-cell mitogen. (iii) Daily administration of CsA to chickens immune to *E. tenella* at the time of secondary inoculation abrogated protective immunity despite the presence of high levels of secretory and circulating antibodies specific for *E. tenella*.

Infection of chickens with *Eimeria* species is accompanied by the production of antibodies (19) and the development of parasite-antigen-specific CMI (17). The role of antibodies in antiparasitic immunity to avian coccidiosis remains contro-

TABLE 2. Effect of CsA treatment on ConA- and LPS-induced lymphoproliferation

Expt	Treatment[a]	[^{3}H]thymidine uptake (Δcpm ± SD)[b] with: ConA	LPS
1	None	31,921 ± 905	19,558 ± 560
	CSA × 1	490 ± 252	26,140 ± 469
2	None	10,734 ± 205	9,781 ± 350
	CSA × 7	3,709 ± 25	9,987 ± 212

[a] Ten 4-week-old SC chickens were treated with CsA once at 1 day before the assay (CSA × 1) or daily for 1 week beginning 1 day before the assay (CSA × 7). No mortality associated with CsA treatment was observed in CsA-treated groups.

[b] Δcpm, Counts per minute with ConA (10 μg/ml) or LPS (100 μg/ml) − counts per minute medium.

versial. Although an earlier study on bursectomized chickens (7) suggested that antibodies were not essential, it failed to rule out the possible role of maternal antibodies in the primary infection with coccidia since chickens were infected at 2 weeks of age. In the present study, 4-week-old chickens were used to rule out any involvement of maternal antibodies. Furthermore, the present investigation indicates that not all hormonally bursectomized chickens lack circulating immunoglobulins. An additional finding that was not discussed in the earlier study is that some of the hormonally treated chickens that are not agammaglobulinemic are more susceptible to coccidiosis compared with nonbursectomized control chickens upon primary infection. Taken together, the results of the present study demonstrate that antibodies play a negligible role in determining disease susceptibility and the development of protective immunity to coccidiosis.

It has been well documented that chickens will develop a good protective immunity against coccidia after infection with the live parasites. Although work on the nature of immunity to coccidiosis and the immune mechanism(s) involved in the mediation of resistance to reinfection have been intensively studied over the past 20 years, the mechanism(s) of the development of protective immunity is not well understood. The importance of CMI has been suggested by the adoptive transfer of protective immunity with immune lymphocytes (23) and the development of potent T-cell responses to sporozoite and merozoite antigens in immune chickens (17). In this study, CsA, a drug with well-characterized cell-mediated immunosuppressive activity (2, 16), was used to define further the involvement of T-cell-dependent mechanisms in avian resistance to coccidiosis. Although the effects of oral CsA treatment on the intestinal immune system of chickens were not investigated, the splenic lymphocyte response to ConA was severely depressed compared with that in untreated control chickens. The importance of a T-cell-dependent mechanism(s) in antiparasitic immunity to coccidia was further demonstrated by the significant numbers of oocysts produced upon secondary infection in chickens treated with CsA. Interestingly, these chickens showed high levels of coccidia-specific secretory IgA and serum IgG, suggesting a minimal role of antibodies in conferring disease resistance. Infecting coccidia do not appear to be directly sensitive to CsA at the physiologically achievable concentration used in the present study since CsA-treated but not untreated chickens shed significant numbers of oocysts upon secondary infection. This is in contrast to malaria, which was shown to be sensitive to CsA (21).

The differential effect of CsA on primary infection with *E. tenella* depending on the length of treatment was not expected but proved to be consistent. Although the mechanism for this phenomenon cannot be explained at present, the results of the present study suggest that CsA exerts different immunopharmacological effects depending on when it is given with respect to antigen priming. Chickens treated with CsA for 1 week beginning 1 day before primary infection were resistant to the secondary infection, suggesting that antigen-specific T-cell priming and clonal expansion occurred despite the CsA treatment. These results seem confusing in view of the fact that CsA is an immunosuppressive drug that is effective in preventing allograft rejection (5, 6), autoimmune reactivity (29), and graft-versus-host disease (28). CsA-mediated enhancement of resistance upon primary infection in CsA-treated chickens may reflect a very complex pattern of events occurring at the induction phase of the immune response toward *Eimeria* species which is somehow altered by CsA. Whether the immune enhancement mediated by CsA is due to the effect of CsA on suppressor cells, similarly suggested in OS chickens (29), or due to inhibition of the anti-inflammatory response mediated by CsA (11) remains to be elucidated. The enhanced resistance conferred by CsA treatment upon primary infection may have an immune basis similar to that induced by CsA pretreatment of other parasitic infections (1, 3, 4). In *Leishmania major* infection in mice (1), CsA treatment had a striking prophylactic effect, and these mice were capable of developing protective immunity. Similar phenomena were described during *Schistosoma mansoni* infections in mice (2, 4). CsA treatment was not only effective in eliminating the infecting worms during the primary infection but also induced a long-term protective effect.

Although CsA has been widely used in patients to prevent allograft rejection, much about the in vivo action of CsA remains unknown. Recent investigations suggest that CsA has somewhat unpredictable effects on the immune systems of animals and humans (13, 26). Some of the effects of CsA in vivo, such as immune enhancement (15, 27) and exacerbation of experimental autoimmune thyroiditis in chickens (29) and *Trypanosoma cruzi* infection in mice (12), need further investigation. The continued in vivo study of CsA and its interactions with the immune system in different experimental systems will shed considerable light on the immunoregulatory pathways which determine the outcome of an immune response toward both infectious and noninfectious antigens.

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Intestinal Infections Of Chickens

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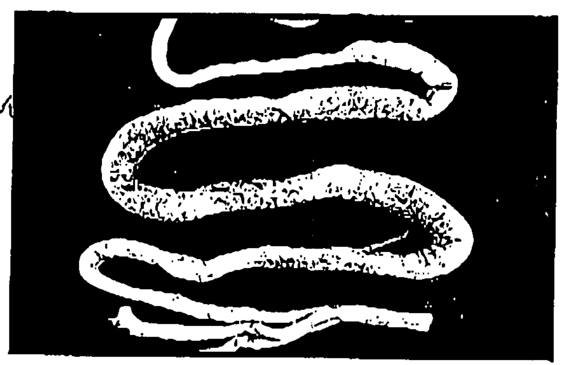
ZOOTECNICA NOV. 2001

Intestinal infections and parasitism are responsible for extensive losses in broilers, immature replacement breeders and commercial egg production flocks. A diverse range of pathogens can damage the mucosa (lining) of the intestinal tract resulting in severe depression in growth in addition to mortality. The intestinal tract is a portal of entry of pathogens into the circulatory system and many bacteria including Salmonella *and* Pasteurella *are transmitted to flocks by ingestion.*

The intestinal tract is responsible for absorption of nutrients. Injury to the mucosa will result in suboptimal growth and feed conversion efficiency. Damage to the mucosa will accelerate the rate of replacement of enterocytes which diverts nutrients from growth to the restoration of the integrity of the intestinal tract. Toxins produced by pathogens can affect intestinal motility resulting in diarrhea. The subsequent loss of fluid will result in dehydration which renders flocks susceptible to heat stress and impaired metabolism. This is reflected in degraded performance including elevated mortality.

Coccidiosis

Coccidiosis is regarded as the most important of the intestinal diseases since without appropriate preventive action, long-term profitable production of commercial poultry is impossible. It is estimated that producers worldwide spend over $200 million annually on anticoccidial medication of feed, vaccines and treatment. In addition, extensive losses occur as a result of overt and subclinical infections which reduce growth rate and feed conversion efficiency. Occasionally outbreaks of clinical coccidiosis occur which may result in mortality of varying severity and stunting in survivors.



Eimeria necatrix. Characteristic swelling of the small intestine

Intestinal coccidiosis is ubiquitous and occurs in all areas where chickens are raised. Generally outbreaks occur as a result of one or more of the following factors:

- Immunosuppression due to exposure to Marek's disease, chick anemia virus or infectious bursal disease.
- Litter moisture exceeding 30%, especially in the vicinity of the drinkers. Saturation of litter following entry of rain into houses may also contribute to high levels of sporulated oocysts, the infective stage of the cycle. Suboptimal ventilation, overstocking and overcrowding at feeders will also result in flocks being extremely susceptible to coccidiosis.
- Suboptimal inclusion of anticoc-

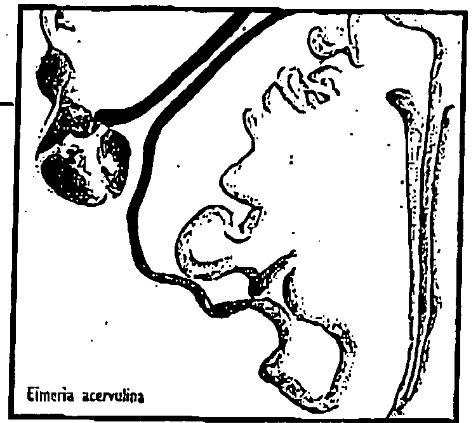
cidials in diets or improper distribution of various compounds in feed may result in outbreaks due to inadequate suppression of the reproductive cycle in the intestine.

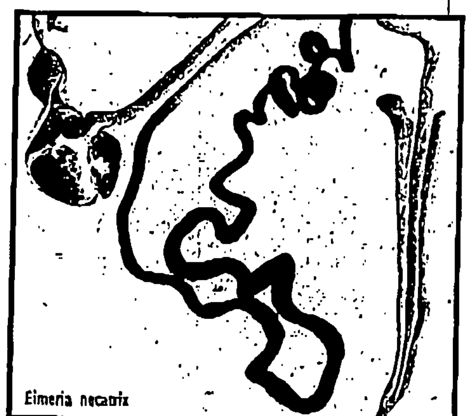
☛ Restricted daily issue and "skip-a-day" feeding programs will predispose replacement breeding flocks to coccidiosis due to variability in feed intake. Starvation among smaller pullets results in litter-eating with a high level of exposure in debilitated birds.

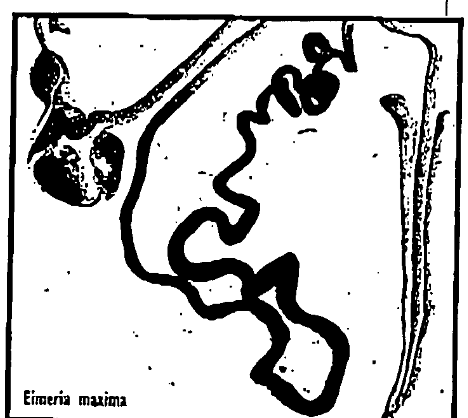
The various *Eimeria* species which infect chickens are specific to regions of the intestinal tract and each is associated with characteristic lesions and severity. Generally there are no specific signs associated with coccidiosis other than an acute onset of depression, which may be accompanied by increasing mortality in the flock. Birds infected with *Eimeria tenella* void blood- stained droppings which are observed on the surface of litter and on perches. Terminally affected birds show extreme paleness of the comb and wattles.
The following lesions are associated with the specific Eimeria species:

☛ *Eimeria acervulina* and *E. mivati*: 1-2 mm white striations are observed through the serosa of the duodenal loop and proximal 10 cm of the small intestine. Depending on the stage of infection, white foci are interspersed with punctate 0.5 mm hemorrhages. The intestinal wall is thickened and edematous.

☛ *E. necatrix*: severe distension of the mid-jejunum occurs with accumulation of blood tinged fluid in the lumen and obvious hemorrhages in the mucosa.


Eimeria acervulina


Eimeria necatrix


Eimeria maxima


Eimeria brunetti

☛ *E. maxima*: the mid- to terminal jejunum is distended with hemorrhages in the mucosa.

☛ *E. tenella*: this infection is characterized by hemorrhagic typhlitis. Acute cases show free blood in the lumen of the cecum progressing to red and gray casts during recovery.

☛ *E. brunetti*: hemorrhages occur in the mucosa of the terminal jejunum and the colon distal to the bifurcation of the ceca.

Coccidiosis is diagnosed on the basis of history, and postmortem lesions. The diagnosis can be confirmed by microscopic examination of scrapings taken from the intestinal mucosa which will reveal characteristic oocysts.
Under commercial conditions it is often necessary to obtain confirmation of a diagnosis by a laboratory, which requires submission of the following specimens:

☛ Representative litter samples for oocyst speciation and quantification.

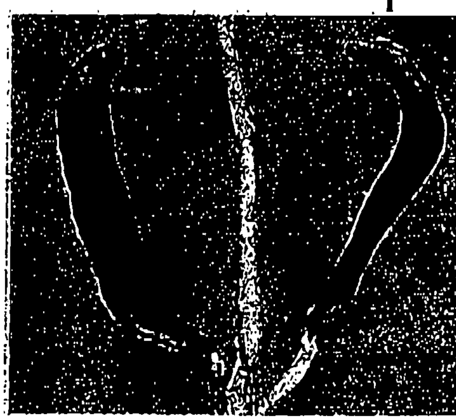
☛ Sections of the affected intestine placed in 10% formalin-saline for histological examination.

☛ Sections of intestine in 5% potassium dichromate solution for culture and speciation of *Eimeria*.

☛ Representative feed samples obtained from unopened bags or directly from the feed bin for assay of anticoccidial content. Feed samples should not be collected from feed pans or troughs, since mechanical separation can occur

resulting in the possibility of biased/ assays.

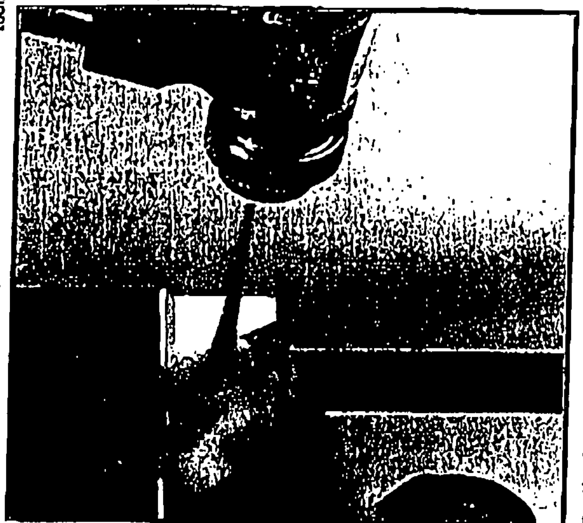
Acute outbreaks of coccidiosis can be treated with 17% amprolium solution at the rate of 1 g/liter in drinking water for 3 to 5 days. Sulfamethazine 12% solution can be administered at the rate of 5 to 10 ml/liter of drinking water for 5 days. Commercial sulfa preparations can be administered in accordance with the manufacturers' recommendations. Withdrawal periods should be followed, to prevent contamination of the food chain. Administration of supplementary water dispersable vitamins A and K may enhance recovery.
Prevention of coccidiosis is based on a coordinated program of flock management, incorporating either anticoccidials or vaccines to immunize flocks prior to exposure. Managemental factors to maintain litter moisture below 30% are essential to prevent coccidiosis. In the event of repeated outbreaks, an evaluation of ventilation, watering systems, function of feeder installations and stocking density should be carried out. Appropriate improvements of obvious deficiencies should be implemented.
Broilers in most countries are fed diets containing an anticoccidial. Coccidiostats suppress the life cycle of *Eimeria* by inhibiting the first and second stage schizonts, the asexual reproductive phases. This class of drugs allows low-level infection but subsequently flocks maintain premunity. In contrasts coccidiocides interdict sporozites, first stage schizonts and merozites, effectively preventing any deleterious effects of exposure. Flocks are however completely susceptible to coccidiosis within five days of removal of a coccidiocide from the diet. Coccidiocides are used in broiler diets when suppression of clinical coccidiosis is required over a growing cycle of up to 50 days. Coccidiostats are fed to floor-reared replacement flocks which must be protected during the early rearing period to develop premunity which will persist through a 50 to 60 week production period on litter.
In the USA and many other countries, a chemical compound such as nicarbazin is included in starter diets during spring, winter and autumn but not during summer due to the deleterious effect of this drug on thermoregulation. During summer months an alternative coccidiocide may be included in diets.
Ionophore anticoccidials are frequently fed during the grower and finisher periods of the broiler cycle but are withdrawn for five days prior to harvest. Occasionally two different ionophores are fed during the starter and the grower/finisher periods in a shuttle program.



To prevent the occurrence of anticoccidial resistance, many operations change anticoccidials after 5 to 8 cycles or when evidence of field exposure occurs.
It is essential to monitor the efficacy of an anticoccidial program by conducting regular feed assays to confirm inclusion and distribution in diets. The intestines of approximate ly 5, clinically normal, sacrificed broilers should be examined at approximately 30 days of age to co firm the absence of infection. This especially important for *E. acervulin* which is generally subclinical, but may result in a 50 to 100 g depression in growth rate during 25 to 35 days of age.

Eimeria tenella

Floor reared replacement breeders are usually protected by inclusion of a synthetic coccidiostat such as amprolium, a level of 125 ppm in diets for the first 6 weeks followed by a reductio to 62 ppm until 16 weeks of age. After this age, pullets and cockerels will be refractory to infection but a proportion of the flock will continue to shed a low level of oocysts to maintain premunity.
Anticoccidial vaccines are gaining popularity due to their effectiveness and relatively low cost in many area of the world. In the USA approxi-



mately 80% of broiler breeder replacements are vaccinated with a suspension of oocysts containing 4 to 5 species. The vaccine suspension is administered in accordance with the manufacturers' recommendations and may be sprayed on feed or administered in drinking water during the first or second day. It is necessary to maintain a moisture content in the litter of approximately 25%, especially in the vicinity of drinkers and feeders during the period 5 to 9 days. This ensures that the first cycle of oocysts derived from the successfully vaccinated birds exposes the entire flock, stimulating premunity.

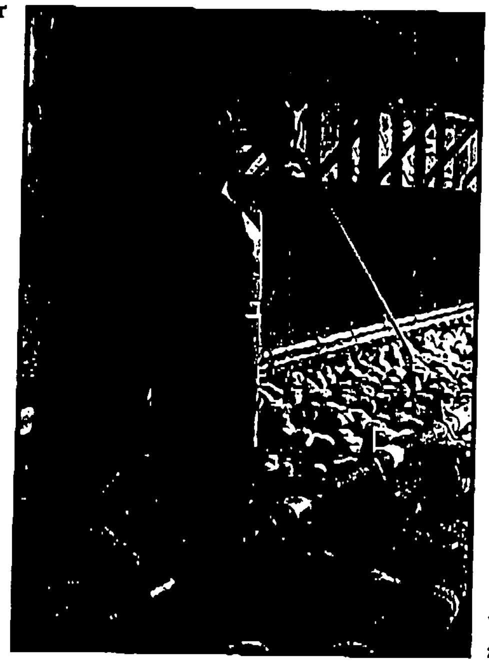
A number of producers now use oocyst vaccines to protect broilers, especially flocks which are grown to roaster weight over a 9 to 10 week cycle. Chicks receive the oocysts suspension in the form of a coarse spray at the hatchery or alternatively the vaccine can be administered in drinking water after delivery to the farm during the first 48 hours of the brooding period.

Attenuated oocyst vaccines are an alternative to the contrived exposure achieved with the commercial oocyst suspensions. The attenuated vaccines comprise either precocious strains which do not undergo the second stage of schizogony and are therefore relatively innocuous or the vaccine strains have been modified by chemical or physical treatment. Attenuated vaccines are more expensive than oocyst suspensions and their use should be justified by field evaluation to confirm that financial benefits are commensurate with the additional cost. Since all oocyst vaccines are sensitive to freezing, care should be taken in the transport and storage of vaccines to ensure that preparations are not exposed to temperatures below 4°C. Occasionally coccidiosis vaccines may be stored in a farm refrigerator adjacent to the freezer compartment for prolonged periods, resulting in decreased potency.

In an attempt to reduce the occurrence of drug-resistant strains of *Eimeria*, some producers administer a highly effective coccidiocide such as diclazuril for two consecutive broiler growing cycles. This will frequently eliminate residual challenge associated with the specific farm. It is important not to prolong administration beyond two cycles in order to limit the emergence of drug resistance. An alternative program which is becoming popular is to intersperse two cycles of treatment with an anticoccidial vaccine followed by resumption of a shuttle program using a chemical coccidiocide and ionophore combination. This approach will displace the drug resistant endemic strains with the vaccine strains, contributing to enhanced efficacy when the use of conventional anticoccidials is resumed for subsequent flocks.

Necrotic enteritis

This condition is becoming prevalent in both broilers and replacement pullets reared on litter. *Clostridium perfringens*, an anaerobic Gram positive organism is responsible for ulceration of the mucosa of the jejunum. Extremely toxigenic strains may result in acute mortality. Necrotic enteritis is multifactorial in etiology and is usually associated with immunosuppression, followed by an environmental stress such as saturation of litter, malfunction of feeding systems, starvation or flooding of the house follow-



The vaccine may be administered either by eye spray at the hatchery or spray application on feed in the broiler house